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Thrombopoietin: In Vitro Predictions, In Vivo Realities

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INTRODUCTION

Belief in a humoral mediator of platelet production developed in the late 1950's, but remained conceptual until thrombopoietin (TPO) was cloned 2 years ago [1–6]. Over the intervening years, many physiologic principles developed, based on the behavior of partially purified preparations of the cytokine in various in vitro and in vivo systems [7,8]. With the availability of the recombinant protein and a number of experimental systems with which to test the hormone, the question of whether the biological activities displayed by TPO in vitro truly represent its in vivo behavior can now be addressed.

THROMBOPOIETIN STIMULATES MEGAKARYOCYTE PRODUCTION IN VITRO

Most early studies using partially purified materials indicated that TPO was primarily if not exclusively a megakaryocyte (MK) differentiation factor [7,8]. Initial studies with the recombinant protein have confirmed it to have potent differentiative activities. Compared to IL-3, IL-6, or IL-11, TPO increases the size of megakaryocytes by 50–70%, increases the geometric mean ploidy by 200–300% over than seen with IL3 or IL-11, and results in cultures predominated by gpIIb/IIIa- and gpIb-expressing cells [9,10]. Megakaryocytes grown in the presence of TPO contain abundant demarcation membranes, platelet-specific granules, and "platelet fields" [10,11]. When cultured on a substratum, the cells form "proplatelet processes" [11,12], thought to be the in vitro equivalent of the cytoplasmic extensions seen between marrow sinusoidal endothelial cells [13], from which megakaryocytes are thought to move into the circulation and from which platelets fragment. And from these cultures one can harvest numerous morphologically normal *platelets which can exteriorize* P-selectin upon exposure to classic platelet agonists [12].

Although not initially thought to support the proliferation of megakaryocytic progenitor cells, TPO is a potent stimulus of megakaryocyte colony growth in vitro. By itself, the recombinant protein supports the proliferation of as many as 75% of all megakaryocyte colony-forming cells in normal marrow [9,14]. Most of the derived colonies contain low-to-intermediate numbers of very large cells, in contrast to colonies obtained in the presence of IL-3 or c-kit ligand (KL), in which up to one third of the colonies contain many megakaryocytes [15]. Nonetheless, TPO acts additively with IL-3, and synergistically with KL, IL-11, and EPO, to enhance the proliferation of CFU-MK [16].

THROMBOPOIETIN STIMULATES PLATELET PRODUCTION IN BOTH MOUSE AND MAN

Given the large body of data relating to the effects of TPO on megakaryopoiesis in vitro, it was not surprising to find that the administration of the protein to normal mice, dogs, baboons, and humans has been associated with profound increases in platelet levels [2,3,9,17,18]. Within 3 days of initiating treatment of mice, and within 7 days in primates, platelet counts begin to increase and are maximal at 4–10 times normal levels just at or beyond termination of its administration. Thrombocytosis is maintained for as long as administration continues, and begins to wane approximately 5–7 days after its discontinuation. The reason for the thrombocytosis is increased platelet production; marrow (and in mice, splenic) megakaryocyte numbers and size are increased up to 10-fold within a week of initiation of treatment [9,17]. These changes begin to subside within 1–2 weeks of cessation. Platelet morphology is essentially normal, except for an increase in the number of "platelet reticulocytes," platelets which bear rough endoplasmic reticulum and stain readily for RNA. Such platelets resemble those found in autoimmune states of platelet destruction. And although platelet function in vitro has been reported to be enhanced by their incubation in TPO [19], platelet function in vivo appears entirely normal, even when tested in a prothrombotic vascular graft model [20].

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IS THROMBOPOIETIN ESSENTIAL FOR MEGAKARYOCYTE AND PLATELET PRODUCTION?

The next question that arises is whether TPO is absolutely essential for megakaryocyte and platelet production. We recently attempted to answer this question with in vitro studies. Over the past several years a number of cytokines have been shown to support the proliferation and differentiation of megakaryocytes in vitro; several of these agents have also been used to stimulate platelet production in vivo. Thus, if TPO is absolutely essential for megakaryocyte and platelet development, these other cytokines must be operating through endogenous TPO in the various culture systems used in past studies. As most studies using IL-3, IL-6, IL-11, and KL have utilized either serum or plasma, which contain the hormone, or culture systems which contain a number of marrow stromal cells, which have been shown to produce TPO, we sought to determine if eliminating TPO from cytokine-stimulated marrow-cell cultures would eliminate megakaryocyte production. Using a soluble form of the Mpl receptor which neutralizes TPO activity, we recently showed that megakaryocytic colony formation in the presence of combinations of KL, IL-6, and IL-11 is obliterated by addition of the soluble receptor [15]. In contrast, although megakaryocytic numbers were reduced by the soluble receptor in colony-forming assays and suspension cultures, they were never eliminated in the presence of combinations of cytokines including IL-3. Moreover, the number of megakaryocyte colonies formed using optimal levels of TPO is always increased if IL-3 is also present, and the number of colonies containing large numbers of megakaryocytes is far greater if IL-3 is included in the culture. These data indicate that IL-3 stimulates a megakaryocytic progenitor cell earlier in its developmental pathway than that supported by TPO. However, none of these IL-3-induced culture results speak to the maturity of the cells. Using suspension cultures we next evaluated the level of megakaryocytic maturity in cultures of marrow cells grown in the presence of IL-3 plus the soluble receptor. None of the cells displayed a ploidy greater than 4N, and the cytoplasm was quite immature; there were no demarcation membranes, no platelet-specific granules, and no platelet fields. The addition of IL-11 to the cultures slightly increased megakaryocyte ploidy and cytoplasmic maturation, but the cells never achieved the developmental potential of cells grown in the presence of TPO alone [21].

The availability of the techniques of homologous recombination has provided a unique opportunity to establish the role of a gene in normal and abnormal physiology. Both the TPO and *c-mpl* receptor genes have now been knocked out [22,23]. The phenotype of both animals is similar; such mice display 5–10% of a normal number of marrow megakaryocytes, and 10–15% of a normal number of blood platelets. In addition, the megakaryocytes present are somewhat smaller than normal. Thus, consistent with our in vitro studies, these results indicate that TPO is the primary determinant of platelet production. The knockout studies also suggest the existence of a low-level, fail-safe mechanism for platelet production. Our data would indicate that IL-3 and IL-11 might comprise that mechanism.

THROMBOPOIETIN IS LINEAGE-DOMINANT, NOT LINEAGE-RESTRICTED

Another recent series of in vitro studies investigated whether TPO may not be as lineage-restricted as initially proposed. There are several theoretical reasons to suspect that this may be the case. First, multiple lines of evidence support the concept of a close relationship between the erythroid and megakaryocytic lineages (reviewed in McDonald and Sullivan [24]). The two share a number of transcription factors. Most of the erythroid cell lines available display, or can be induced to display features of megakaryocytic differentiation. A similar tenet holds for cell lines initially thought to be exclusively megakaryocytic. And a transgenic mouse that carries a suicide gene under the control of a "megakaryocyte-specific" promoter (gpIIb) causes failure of both platelet and red-cell production upon activation [25]. A second line of evidence suggesting a more widespread physiologic role of TPO is inferred from the pathology of MPLV, the murine myeloproliferative leukemia virus whose identification ultimately led to the cloning of TPO. The disease is characterized by a panmyeloid expansion leading to transformed cells of erythroid, myeloid, and megakaryocytic lineages [26]. This murine retrovirus acquired its oncogenicity by capture and mutation of the gene encoding the TPO receptor, *c-mpl* [27]. Thus, the panmyeloid effects of the virus clearly indicate that a constitutively active Mpl receptor *can* stimulate all hematopoietic cell lineages. Of course, this does not necessarily prove that the normal receptor does so.

Several groups have now studied the lineage specificity of TPO in vitro. We found that adding TPO to cultures containing EPO and IL-3 increased the number and size of BFU-E which developed from marrow cells [28]. Moreover, the cytokine was found to augment the generation of CFU-E in a two-stage EPO-containing *suspension* culture system. Using plucked early BFU-E precursors, Kobayashi et al. [29] found similar results: the presence of TPO approximately tripled the number of BFU-E that developed in low-serum, low oxygen-containing cultures. We found a similar synergistic interaction between TPO

and KL during granulocyte-macrophage colony development.

To investigate whether TPO acts on even more pluripotent cells, Ku et al. [30] studied its effects on the primitive hematopoietic CFU-blast cell population. They found that TPO acts in synergy with KL or IL-3 to enhance the formation of colonies from primitive murine hematopoietic cells isolated from marrow following the administration of 5-fluorouracil [30]. We recently studied the effect of TPO on purified hematopoietic stem cells. Using a highly selected stem-cell population from which long-term hematopoietic reconstitution routinely occurs with 10 cells, and occasionally with a single cell, we tested single sorted cells for direct effects of TPO. As happened for Ku et al. [30], by itself the hormone failed to support the proliferation of any of these cells. However, in the presence of either IL-3 or KL, TPO sped entry of cells into the cell cycle, increased the number of cells that ultimately started to divide, reduced the mean cell cycle time, and led to greater numbers of committed hematopoietic progenitors of all cell lineages compared to cultures with these other cytokines but without TPO [31]. Based on these in vitro studies, in addition to its effects on megakaryocyte biology, TPO also appears to exert a direct effect on primitive stages of hematopoiesis.

Two lines of in vivo evidence support the concept that TPO can act as a pleotropic hematopoietic regulator. Recently, levels of hematopoietic progenitor cells in the *c-mpl* and TPO knockout mice were analyzed. Marrow and splenic erythroid, myeloid, and mixed hematopoietic lineage progenitor cells were all substantially reduced in both settings [22]. Results from preclinical trials of TPO have led to similar conclusions. When administered to normal mice, and to a lesser extent in normal nonhuman primates, TPO modestly expands the numbers of marrow and splenic hematopoietic progenitor cells of multiple cell lineages [28]. However, these effects do not result in an increase in red cell or leukocyte production, as the primary regulator of these lineages (EPO and G-CSF respectively) are not increased in normal animals. In contrast, when administered to pancytopenic animals, hematopoietic progenitor recovery of all lineages is greatly accelerated, and because of the increased levels of erythroid and myeloid cytokines in these settings, red cell and leukocyte recovery is greatly improved [28]. Thus, taken together, these data suggest that the hematopoietic response to TPO will likely be greater than initially anticipated.

WHAT THE FUTURE HOLDS

Thrombopoietin has evolved from concept to reality in 2 short years. Its physiological properties have been extensively studied; the results have both supported old concepts and generated new ones. The most anticipated future results will come from clinical studies, currently underway. However, as the cytokine reaches the stage of clinical investigation, it would seem prudent to keep the lessons derived from in vitro studies of TPO in mind when designing and interpreting the results of clinical trials.

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